

Extracellular matrix and hormones transcriptionally regulate bovine β -casein 5' sequences in stably transfected mouse mammary cells

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ABSTRACT Milk protein regulation involves synergistic action of lactogenic hormones and extracellular matrix (ECM). It is well established that substratum has a dramatic effect on morphology and function of mammary cells. The molecular mechanisms that regulate the ECM- and hormone-dependent gene expression, however, have not been resolved. To address this question, a subpopulation (designated CID 9) of the mouse mammary epithelial cell strain COMMA-1D has been developed in which more than 35% of the cells express β -casein, form alveoli-like structures when plated onto a reconstituted basement membrane, and secrete β -casein unidirectionally into a lumen. These cells were stably transfected with a series of chloramphenicol acetyltransferase (CAT) fusion genes to study transcriptional regulation of the bovine β -casein gene. The expression of CAT in these lines demonstrated a striking matrix and hormone dependency (>150 -fold induction in some cases). This regulation occurred primarily at the transcriptional level and was dependent on the length of the 5' flanking region of the β -casein promoter. Both matrix and hormonal control of transcription occurred within at least the first 1790 base pairs upstream and/or 42 base pairs downstream of the transcriptional initiation site. The ECM effect was independent of glucocorticoid stimulation. However, prolactin was essential and hydrocortisone further increased CAT expression. Endogenous β -casein expression in these lines was similar to that of the parent CID 9 cells. Our data indicate the existence of matrix-dependent elements that regulate transcription.

Mammary epithelial cells provide a prime example for the dramatic effect of extracellular matrix (ECM) on morphology (1–3) as well as milk protein gene expression (2, 4–7). The molecular mechanisms involved in this interaction, however, are far from understood.

Two approaches have been taken to address this problem: (i) To determine the nature of ECM–mammary cell interaction at the cell surface (8) and (ii) to determine the nature of cis- and trans-acting sequences and factors involved in transcriptional regulation. The absence of functional cell lines for stable transfection of chimeric genes has been a major impediment in the latter approach. While transgenic animals have been used for studies of hormonal regulation (9), the role of the ECM in tissue-specific gene expression cannot be easily studied in such models.

With the availability of the functional COMMA-1D mammary epithelial cell strain derived from midpregnant mice (10), a number of laboratories have attempted to study the regulatory sequences of milk protein genes in response to lactogenic hormones. Some earlier attempts to transfect casein constructs failed, as the transfected cell bypassed hormonal regulation (11). In HC11 cells, a cloned derivative

of COMMA-1D cells, Doppler *et al.* (12, 13) succeeded in dissecting aspects of the hormone-responsive cis-acting elements of the β -casein gene. These cells, however, have lost their ability to respond to substrata and therefore are not applicable for deciphering the molecular mechanisms of ECM action. Transient transfection into primary mouse cultures has also been attempted. Moderate hormone- and substratum-dependent modulation has been demonstrated recently (14). However, in addition to the transient nature of these studies, primary cultures do not easily recover after transfection and the level of DNA uptake is not reproducible from experiment to experiment (ref. 14; C.S., unpublished results).

We have therefore produced a cell strain that is responsive to both hormones and substrata and that could be stably transfected. In this paper, we describe the isolation of these cells and report that expression of bovine β -casein chloramphenicol acetyltransferase (CAT) fusion genes is dependent on the length of the 5' bovine β -casein promoter and that maximal expression is completely dependent on both ECM and prolactin. This system allows us to compare regulation of the transfected genes with expression of the endogenous β -casein.

MATERIAL AND METHODS

Cells and Medium. Parent COMMA-1D (10) and CID 9 cells were grown in DMEM/F12 (1:1; GIBCO) containing 5% heat-inactivated fetal calf serum (FCS), gentamicin (50 μ g/ml), and insulin (Sigma; 5 μ g/ml). For hormonal and ECM-dependent milk protein induction, the cells were plated on plastic dishes or reconstituted basement membrane EHS (15) at 8×10^4 cells per cm^2 in the presence of 2% FCS and insulin. Hydrocortisone (Sigma; 1 μ g/ml) and/or ovine prolactin (National Institutes of Health; 3 μ g/ml) was added as indicated. Twenty-four hours after plating, the medium was switched to 0% FCS.

Bovine β -Casein Gene Cloning and β -Casein–CAT Gene Fusion. The bovine β -casein (β cas) gene was isolated from a library of bovine genomic DNA fragments (16) cloned in the λ phage vector EMBL4. Five clones were isolated that contained overlapping bovine genomic DNA fragments that together spanned 27 kilobases (kb) of the bovine genome. The cloned DNA contained the bovine β -casein transcription unit on a 9-kb *EcoRI* fragment as well as 4 kb of 5' flanking and 14 kb of 3' flanking DNA (see Fig. 5). The restriction map of this region and partial nucleotide sequence of the 5' flanking and transcribed region (data not shown) agree well with those previously reported for the bovine β -casein gene (17). Details of the isolation of the clones and the structure of their bovine genomic inserts will be described elsewhere.

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Abbreviations: ECM, extracellular matrix; CAT, chloramphenicol acetyltransferase.

Each β cas genomic fragment of these constructs contains a unique 5' terminus and is fused to CAT at position +42 (relative to the transcription start site) in the first, noncoding β cas exon. These expression cassettes were assembled in pMON3605 (18), a pUC18-based vector containing the simian virus 40 late transcription terminator. The β cas fragment extending from the *Xba* I site at -791 to +42 of exon 1 was synthesized by polymerase chain reaction (GeneAmp kit, Perkin-Elmer/Cetus), which also served to create the *Bam*HI site in exon 1. Similarly, the β cas-3815+42 promoter fragment was synthesized by polymerase chain reaction. β cas promoter fragments between -791 and -2605 were obtained from the cloned genomic DNA and subcloned into β cas-791+42 to create β cas-1790+42 and β cas-2605+42. The *Bam*HI fragment of the bacterial CAT gene [pCM4; Pharmacia (19)] was cloned into the unique *Bam*HI sites that lay between the β cas and simian virus 40 DNA fragments of each plasmid to give the final β cas/CAT/simian virus 40 constructs (see Fig. 5). These constructions were made by standard techniques (20).

Transfection of CID 9 Cells and CAT Assays. Calcium phosphate precipitation was carried out according to the protocol of Gorman (21) using 40 μ g of DNA per 100-mm dish. G418 (GIBCO; 400 μ g/ml) selection was started 48 hr after transfection. Surviving colonies were pooled and expanded for stocks and assays. For CAT assays, differentiated cells were harvested by digesting the EHS with dispase (Collaborative Research). CAT assays were carried out in a two-phase diffusion system (22) using [14 C]acetyl coenzyme A (NEN; 4 mCi/mmol; 1 Ci = 37 GBq) at 0.1 μ Ci per assay. The reactions were carried out at room temperature. CAT activity was calculated as substrate conversion per min per μ g of protein.

RNA and Northern Blot Analysis and Protein Analysis. Total RNA was isolated as described (23). Random-primed probe (24) was prepared by using gel-purified insert sequences from a plasmid containing 540 base pairs (bp) of β -casein cDNA kindly provided by J. M. Rosen (Baylor College, Houston, TX). For protein analysis, cell, luminal, and medium fractions of labeled proteins were prepared as described (3, 25). The total incorporated radioactivity was calculated from trichloroacetic acid-precipitable counts from all three fractions and aliquots representing equal numbers of trichloroacetic acid-precipitable cpm were used for immunoprecipitation.

Nuclear Run-On. *In situ* transcription assays were performed according to a modified protocol of Ucker and Yamamoto (26). Primary cells were grown for 6 days under the hormone and substrata conditions as indicated. Cells still attached to the plates were permeabilized with digitonin (Sigma; 3 mg/ml) and processed as described (26). Sarcosyl (0.2%) was used to block initiation of chains. The elongation was performed at 25°C for 20 min and terminated by adding 4 M guanidinium thiocyanate/25 mM sodium citrate, pH 7.0/0.5% sarcosyl. Total RNA was then isolated as described (23) and 1.5×10^6 cpm of labeled RNA was hybridized overnight with DNA (10 μ g per slot) spotted to a nylon membrane (Hybond-N, Amersham).

RESULTS

Generation of an Enriched Population (CID 9) of β -Casein-Producing Cells. A confluent culture of COMMA-1D cells was treated with trypsin for different lengths of time. At each time point, detached cells were plated onto EHS and cultured for 3 days in medium containing all three lactogenic hormones. The cells cultured on EHS were then treated with trypsin again and dissociated to a single cell suspension. Cytospins of these cells were analyzed with a monoclonal antibody directed against β -casein. Cells with a greater

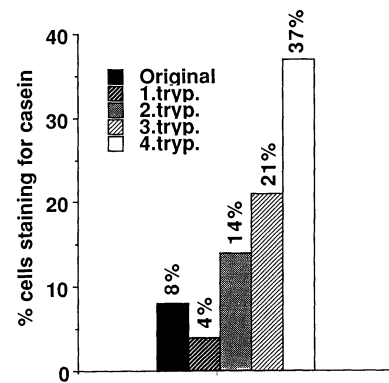


FIG. 1. Enrichment of functional cells by differential trypsin treatment. COMMA-1D cells were treated with trypsin for four different lengths of time. At each time point (1.tryp, 2.tryp, 3.tryp, 4.tryp) the detached cells were removed. These four subpopulations and the starting culture were plated on EHS for 3 days with all three lactogenic hormones and analyzed with a monoclonal antibody against β -casein to determine the percentage of casein-producing cells. The subpopulation in which 37% of the cells stained for β -casein was designated CID 9.

resistance to trypsin treatment had a much higher frequency of β -casein expression. Passage 10 cultures of COMMA-1D contained 8% β -casein-producing cells. The first trypsin-treated subpopulation had a reduced frequency of 4% β -casein production (probably due to fibroblast-like enrichment). Increasing the length of trypsin treatment released subpopulations progressively enriched in casein-producing cells; in the last fraction, 37% of cells stained for β -casein (Fig. 1). This subpopulation (designated CID 9), enriched in casein-producing cells, formed alveoli-like structures on EHS with a similar morphology to primary mouse mammary cells (Fig. 2). In addition, the level of casein mRNA was only slightly reduced in CID 9 cells compared to t_0 cells or primary cultures produced from 15-day pregnant mouse mammary gland (Fig. 3). In CID 9 cells, β -casein expression was completely dependent on substrata. Little or no β -casein mRNA was detectable in cells grown on plastic for 6 days in the presence of all three lactogenic hormones (Fig. 3).

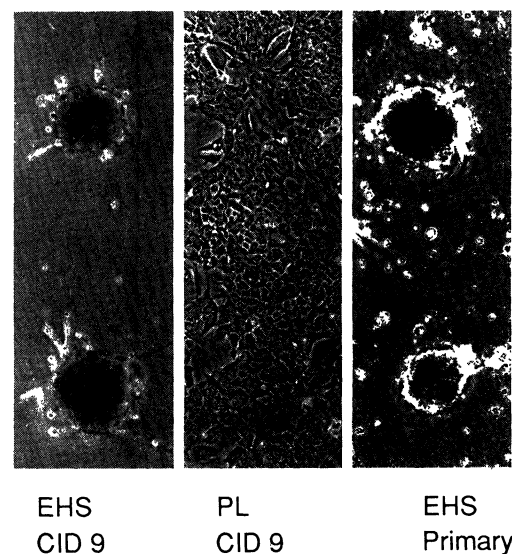


FIG. 2. Morphological comparison between CID 9 and primary cultures grown on EHS. CID 9 and primary cells were grown on EHS for 6 days in the presence of all three lactogenic hormones. CID 9 cells were also cultured on plastic (PL).

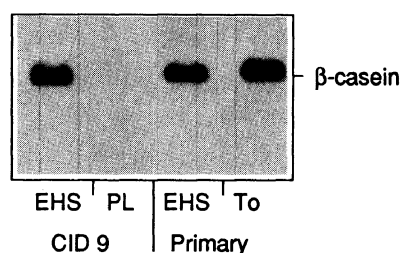


FIG. 3. Comparison of β -casein mRNA levels in CID 9 cells and primary cells from 15-day pregnant mice. CID 9 and primary cells were maintained for 6 days in defined medium containing all three lactogenic hormones. RNA (5 μ g) from cells on EHS or plastic (PL) and time zero (To) of primary cells were separated on a Northern blot and probed with a cDNA probe specific for mouse β -casein.

The profile of milk protein synthesis confirmed the substratum dependency of differentiation (Fig. 4). Cells cultured in EHS, but not on plastic, expressed large amounts of caseins and transferrin. Furthermore, the pattern of milk protein secretion was analogous to primary culture (3)—i.e., there was bidirectional secretion of transferrin and unidirectional secretion of β -casein into the lumen. Little or no β -casein could be detected when cells were cultured on plastic.

Expression of Bovine β -Casein-CAT Fusion Genes in Stably Transfected CID 9 Cells. A series of bovine β -casein-CAT fusion genes were constructed with various lengths of 5' flanking DNA sequences attached at nucleotide +42 of the first, noncoding bovine β -casein exon to CAT (Fig. 5). These plasmids were cotransfected with pSV2neo to confer antibiotic resistance. After G418 selection, between 100 and 500 colonies were pooled and cultured on different substrata in defined medium containing different hormone combinations. Cells transfected with $b\beta$ cas-791+42/CAT had an average CAT activity of 0.7 (relative substrate conversion per min per μ g of protein) (Fig. 6, EHS ihp). Using $b\beta$ cas-1790+42/CAT, the CAT activity of the lysates was 9.4; a 17.7-fold increase (Table 1, average increases calculated from each set of transfections). The CAT activity of the lysates was 37.5 with the $b\beta$ cas-2605+42/CAT construct, indicating an additional 3.7-fold increase compared with the $b\beta$ cas-1790+42/CAT construct or a 48-fold increase compared with the $b\beta$ cas-791+42/CAT construct. When cells were transfected with $b\beta$ cas-3815+42/CAT, the activity dropped to 14.1 (Table 1 and Fig. 6). These data indicate that with optimal external

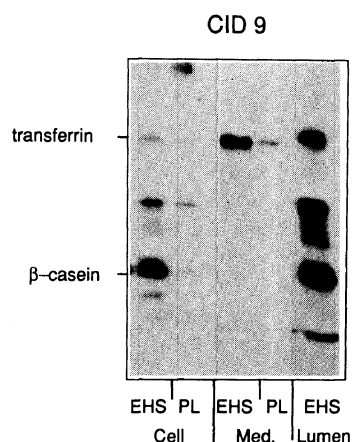


FIG. 4. Influence of ECM on the synthesis and secretion of milk proteins in CID 9 cells. CID 9 cells were grown for 6 days on EHS or plastic (PL) in defined medium containing all three lactogenic hormones and labeled for 4 hr. The cellular fraction, the fraction secreted into the lumen, and the fraction secreted into the medium were immunoprecipitated with a polyclonal antibody against mouse milk and separated on SDS/12% polyacrylamide gel. β -Casein and transferrin are indicated.

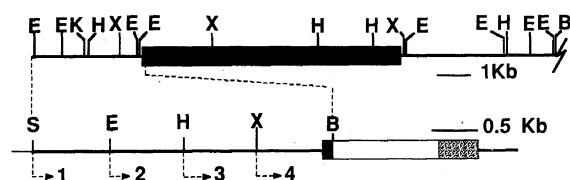


FIG. 5. Restriction map of the bovine β -casein gene and $b\beta$ -cas fusion genes. Flanking regions are represented by heavy lines. The 9-kb transcription unit is indicated by the solid box. The direction of transcription is left to right. The isolated DNA extends \approx 10 kb downstream of this map. The $b\beta$ -cas-CAT fusions are shown below in an expanded scale. 1, $b\beta$ cas-3815+42/CAT; 2, $b\beta$ cas-2605+42/CAT; 3, $b\beta$ cas-1790+42/CAT; 4, $b\beta$ cas-791+42/CAT. Restriction sites indicated on the fusion maps either are not present in the genome or form the boundaries of the $b\beta$ -cas DNA. Open box, CAT DNA; stippled box, simian virus 40 late terminator; the thin line to the left of the $b\beta$ -cas constructs indicates a fragment from the left arm of EMBL4. E, *EcoRI*; B, *BamHI*; H, *HindIII*; K, *Kpn I*; X, *Xba I*; S, *Sal I*.

regulators β -casein CAT expression increases to a maximum near -2605 bp from the transcription initiation site.

The level of induction with both the $b\beta$ cas-3815+42/CAT and the $b\beta$ cas-2605+42/CAT construct was 37.9-fold higher on EHS than on plastic. Induction reached 167-fold for the $b\beta$ cas-1790+42/CAT construct (Fig. 6). The $b\beta$ cas-791+42/CAT still showed some ECM dependency (1.8-fold induction) but the induction was strongly reduced. A similar ECM effect was observed when cells were grown in the absence of hydrocortisone, but the level of induction was reduced because of the decreased activity on EHS itself or was not calculable because the CAT activity on plastic was not significantly different from zero (Table 1).

To examine whether transfected bovine β -casein-CAT fusion genes were hormonally regulated, we plated the stably transfected cells in defined medium containing different hormone combinations (Table 1). Hydrocortisone-induced CAT expression showed a 1.7-fold induction for the $b\beta$ cas-3815+42/CAT construct, a 7.4-fold induction for the $b\beta$ cas-2605+42/CAT construct, a 5.5-fold induction for the $b\beta$ cas-1790+42/CAT construct, and an 8.1-fold induction for the shortest $b\beta$ cas-791+42/CAT construct (Fig. 6, EHS ihp

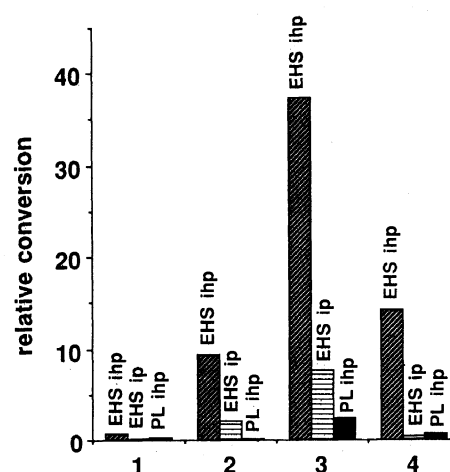


FIG. 6. Effect of β -casein promoter length, substrata, and hormones on CAT expression in transfected CID 9 cells. CID 9 cells transfected with: 1, $b\beta$ cas-791+42/CAT; 2, $b\beta$ cas-1790+42/CAT; 3, $b\beta$ cas-2605+42/CAT; 4, $b\beta$ cas-3815+42/CAT. Cells were plated on EHS or plastic (PL) and maintained for 6 days in culture. CAT activity is expressed as relative conversion of the substrate per μ g of protein per min. The slope was calculated from a series of time points. The means are given after subtraction of background obtained from nontransfected cells and are averages (where means of two sets were available) of total activities as outlined in Table 1. Hormones are indicated in lowercase letters as follows: i, insulin; h, hydrocortisone; p, prolactin.

Table 1. Total activity of CAT expression from individual sets of transfected CID 9 cells under various hormone and substratum conditions

	EHS ihp	PL ihp	EHS ip	PL ip	EHS ih
–3815CAT 1.set	13.63	1.12	6.58	0.17	*
–3815CAT 2.set	14.62	0.23	10.21	0.73	0.02
–2605CAT 1.set	59.70	4.59	4.46	0.88	*
–2605CAT 2.set	15.06	0.24	10.58	0.55	0.10
–1790CAT 1.set	13.83	0.05	1.50	*	*
–1790CAT 2.set	4.93	0.14	2.66	0.48	*
–791CAT 1.set	0.92	*	0.06	*	*
–791CAT 2.set	0.46	0.27	0.29	0.35	*

The means are after subtraction of background obtained from nontransfected cells and express relative conversion per μg per min. Substrata used are indicated in capital letters [EHS and plastic (PL)]. Hormones are indicated in lowercase letters (i, insulin; h, hydrocortisone; p, prolactin). EHS i, PL i, and PL ih conditions showed no activity with all four constructs.

*No activity.

versus EHS ip). When prolactin was omitted, no significant CAT expression was detected with any of the constructs (Table 1).

Furthermore, the regulation of β -casein 5' flanking sequences was cell type specific since little CAT activity could be observed in CHO cells (data not shown). Hormone and substratum effects were specific for the β -casein–CAT constructs since transfection of CID 9 cells with a simian cytomegalovirus promoter–CAT construct (27) resulted in high expression of CAT that was not regulated by ECM and hormones (data not shown).

Endogenous β -Casein mRNA in Stably Transfected CID 9 Cells. To compare the regulation of the endogenous β -casein gene in transfected cells with the CID 9 cells, total RNA was isolated from cells grown on either EHS or plastic. The β -casein expression in the transfected cells was still strongly regulated by ECM and the total level of β -casein mRNA was only slightly lower than in the parent CID 9 cells (Fig. 7).

Transcription Assays. As an attempt to confirm the transcriptional level of β -casein regulation observed in the results described above, we compared the rates of transcription on primary cells grown for 6 days on different substrata and with different hormone combinations. Hybridization of the labeled RNA to four separate regions of single-stranded genomic DNA (cloned in M13 and covering nearly the whole mouse β -casein gene; kindly provided by J. M. Rosen) indicated a high transcription rate in cells grown on EHS in the presence of all lactogenic hormones (Fig. 8). However, in cells cultured on plastic with all three hormones, transcription was strongly reduced and was extremely low for cells on

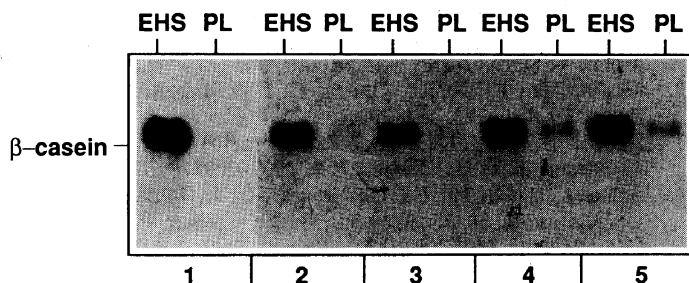


FIG. 7. Comparison of endogenous β -casein mRNA levels among transfected CID 9 cells. Lanes: 1, CID 9 cells; 2–5, CID 9 cells transfected with b β cas-3815+42/CAT (lane 2); b β cas-2605+42/CAT (lane 3); b β cas-1790+42/CAT (lane 4); b β cas-791+42/CAT (lane 5). Cells were plated on EHS or plastic (PL) and maintained for 6 days in the presence of all three lactogenic hormones. Total RNA (5 μg) was separated on a Northern blot and probed for the presence of β -casein mRNA.

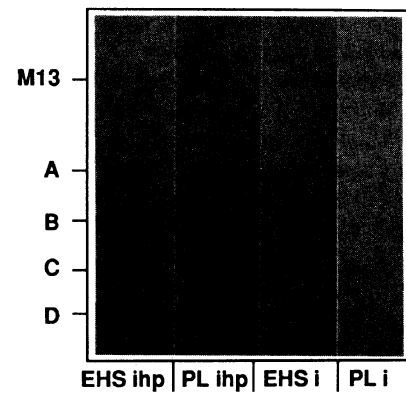


FIG. 8. Influence of substrata and hormones on transcription rates. Nuclear run-on of β -casein RNA in primary cell grown on EHS in the presence of insulin, hydrocortisone, and prolactin (EHS ihp) or in the presence of insulin alone (EHS i) were compared with the transcription rates of cells grown on plastic (PL) with the same hormone combination. Four pieces of single-stranded DNA covering nearly the whole β -casein gene from the 5' (band A) through the 3' (band D) termini were used as probes (28). Control hybridization with M13 alone is indicated.

either substratum with insulin alone. No hybridization could be detected with the M13 control.

DISCUSSION

The influence of a spectrum of environmental regulators on tissue-specific gene expression is increasingly appreciated (29). It is therefore critical that cells in culture are not only provided with an appropriate microenvironment, but also that they are shown to respond to external signals so that correct molecular mechanisms for gene expression may be deciphered.

Conditions have been defined under which primary cells from mid-pregnant mouse mammary gland could remain functional in culture (1–3, 5, 7, 8, 30, 31). While these cultures are ideal for studying some aspects of gene regulation, they are difficult to use for molecular studies with transfected genes. This is because culture conditions have been defined for differentiation and not for growth. Therefore primary cells cannot be stably transfected with the desired DNA constructs to elucidate the nature of regulatory sequences.

The COMMA-1D cell strain (10), while responsive to hormones and ECM in early passage, loses this response rapidly in a later passage (32). In addition, it is extremely heterogeneous with the number of β -casein-producing cells, also declining with passage. However, this cell strain did serve as an important starting point for developing a subpopulation that would behave functionally and morphologically in a similar fashion to primary cells. The CID 9 subpopulation described in this paper satisfies most of these criteria; the cells retain their ability to differentiate and function even after a selection and proliferation period of >4 weeks after transfection. The HC11 cell line (33), a cloned derivative of COMMA-1D cells, while useful for hormonal regulation (12, 13), cannot be used for ECM studies. These cells require high density for function but are not ECM responsive.

Having established CID 9 cells as a functional proliferating cell strain, one of our first goals was to narrow down the sequence requirements for β -casein regulation. The stepwise reduction in activity between nucleotides –2605 and –791 points to the existence of multiple regulatory elements within the region between +42 and –2605.

It is well known that substratum has dramatic effects on function and morphology of different cell types, including mammary cells (34–37). Our data demonstrate the existence

of matrix-dependent regulatory elements located within the -1790-bp 5' flanking region or the +42 bp included downstream of the initiation site. Thus far, it is not clear to what extent the ECM-induced expression can be dissociated from the prolactin-induced expression because prolactin was essential for any promoter activity. However, since conditions in which hydrocortisone was omitted still show a clear ECM effect, the substratum effect is at least glucocorticoid independent.

As expected, the hormonal milieu also influenced the expression of β -casein-CAT fusion genes. The combination of insulin hydrocortisone and prolactin is necessary for maximal induction. The reduction of activity when hydrocortisone was omitted is consistent with the results of others (12, 13, 38). The level of transcriptional regulation by hydrocortisone may not be as strong as the level of ECM regulation. The relatively small effect of hydrocortisone on transcription of the β -casein fusion gene cannot completely account for the 30- to 40-fold effect of hydrocortisone on the mRNA level. The complete disappearance of activity in the absence of prolactin is not surprising since it is known that maintenance of glucocorticoid receptor in primary mouse mammary cultures requires the presence of both hydrocortisone and prolactin (39). Thus, removal of prolactin may result in loss of the glucocorticoid stimulation and thereby down-regulate β -casein expression.

The results described above indicate a strong transcriptional regulation of the transfected genes by ECM and hormones. Since both the endogenous β -casein mRNA and CAT activity were strongly reduced in cells maintained on plastic most of the regulation by ECM must occur at the transcriptional level. This is in contrast to earlier nuclear run-on studies carried out with COMMA-1D and primary cells (7, 38). Since in these experiments there was only a 2- to 3-fold difference in β -casein transcription between cells grown on plastic and cells grown on EHS, the much bigger difference in the steady-state level of mRNA was thought to be mainly posttranscriptional. These results were based on the use of double-stranded cDNA probes that represented the last three exons of the casein gene. Recently, Goodman and Rosen (28) have reported that the use of single-stranded probes reveals a large transcriptional component in β -casein gene regulation in COMMA-1D and HC11 cells on floating collagen and plastic. To ensure that this finding is physiological and is also found on EHS, we investigated the ECM and hormonal regulation on sense transcription of β -casein in primary cultures. The transcription rate on plastic with all three hormones was greatly reduced. In the presence of insulin alone, transcription was extremely low even for cells grown on EHS. However, down-regulation of transcription on plastic is not a universal phenomenon. The transcription of a tissue inhibitor of metalloproteinases, for example, is low on EHS but is strongly up-regulated on plastic in mammary cells (R. Talhouk and C.S., unpublished data). Thus, while posttranscriptional events should not be ruled out completely, this component of β -casein regulation may not be as large as previously thought.

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